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Brief Report

In vivo and *in vitro* expression analysis of the RNA-dependent RNA polymerase of Citrus tristeza virus

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Summary

Expression of the RNA-dependent RNA polymerase (RdRp) of Citrus tristeza virus (CTV) was studied *in vivo* and *in vitro* using a polyclonal antiserum raised against the recombinant CTV-RdRp protein. Although a 57-kDa CTV-RdRp was expected to be expressed by a +1 translational frameshift at the carboxyl terminus of a 400-kDa polyprotein, a 50-kDa protein was detected in CTV-infected but not in healthy citrus tissue by Western blot. This suggests that the RdRp was cleaved from the CTV polyprotein. The 50-kDa protein was present in both the cytoplasmic and membrane fractions, but it accumulated mainly in the membrane fraction, where most of the replication-associated proteins of RNA viruses are found. When the expression of a cloned CTV-RdRp gene encoding a 60-kDa fusion protein was studied *in vitro* in a rabbit reticulocyte lysate system, two smaller proteins of about 50 kDa and 10 kDa were detected in addition to the expected 60-kDa protein. All three proteins were immunoprecipitated with the anti-CTV-RdRp serum, suggesting that the 50-kDa and 10-kDa proteins were fragments of the 60-kDa CTV-RdRp fusion protein. When the expression of the RdRp was analyzed at different times during *in vitro* translation, the 60-kDa and 50-kDa proteins were detected at all time points, and a small amount of the 10-kDa protein was detected after 30 min of translation. These results suggest that the CTV-RdRp may also be cleaved *in vitro* in the rabbit reticulocyte lysate.

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Despite wide variation in morphology, genome organization and sequences in their structural proteins, all positive-stranded RNA viruses encode an RNA-dependent RNA polymerase (RdRp) [13]. The RdRp functions as the catalytic subunit of the viral replicase and is required for transcription and replication of the viral genome [6]. Although the RdRps are universally conserved among different groups of RNA viruses, these viruses use a variety of mechanisms for expression of their RdRps. In some viruses, the RdRp is produced as a single

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peptide, but in others it is expressed as a polyprotein containing the RdRp and one or more other viral proteins. The expression of the RdRp is controlled and regulated in different viruses by various mechanisms, such as proteolytic processing, translational read-through or frameshifting [11].

Isolation of viral RdRps from infected host cells is usually difficult due to a low level of expression and/or accumulation. Consequently, most viral RdRps have been identified based on sequence analysis of conserved domains [26]. However, for some animal and plant viruses, the catalytic activity of the RdRp has been demonstrated in partially purified extracts isolated from infected host cells [17]. But isolation of partially purified active RdRp from infected plants has been unsuccessful for most plant viruses due to host characteristics, low expression level, and/or poor solubility of the purified RdRp itself [17].

Difficulties associated with the purification of the RdRp from the infected host cell may be overcome by expression of the RdRps in *E. coli*. Enzymatically active RdRps of poliovirus [30], hepatitis C virus [5, 22], tobacco vein mottling virus [19] and bamboo mosaic virus (BaMV) [21] have been expressed in *E. coli*, and their function and catalytic activity have been characterized. Some other viral RdRps were expressed in *E. coli*, and the products were used for production of antibodies specific for the viral RdRps. These antibodies were later used for detection, identification and functional dissection of RdRps of RNA viruses in the infected host tissue [18, 35].

Citrus tristeza virus (CTV) belongs to the genus *Closterovirus* in the family *Closteroviridae* [4]. It has long thread-like, flexuous, filamentous particles about 2000 nm by 11 nm [3]. Virions of CTV consist of a single-stranded positive-sense RNA molecule encapsidated by two capsid proteins (CPs). The 25-kDa major CP encapsidates about 95% of the genome, and the remaining portion of the genome is encapsidated by the 27-kDa minor CP, on one end of the virion [10]. Published complete genome sequences of seven different CTV isolates with different biological properties and geographical origins have shown that the genome of CTV ranges from 19226 to 19306 nt and is organized into 12 open reading frames (ORF) potentially encoding 17 protein products, plus the 3′ and 5′ untranslated regions (UTR) [1, 20, 25, 27, 31, 36, 37]. Analysis of replication using an infectious cDNA clone of CTV revealed that the ORFs 1a and 1b and the 5′ and 3′ UTRs are necessary and sufficient for genome replication in *Nicotiana benthamiana* protoplasts [32].

In recent years, significant progress has been made in the analysis of the CTV genome [1, 20, 25, 28, 31, 36, 37], the molecular basis and elements involved in genome replication [2, 15, 16, 33], and the functional characterization of some CTV genes [14, 23, 24, 28, 34]. However, the characterization of the genes encoding the replication-associated proteins, including the RdRp, has not advanced. Sequence analysis of the CTV genome has indicated that the putative 57-kDa protein encoded by ORF1b contains sequences similar to typically conserved motifs of RdRps of positive-stranded RNA viruses [20]. It has been proposed that the CTV-RdRp is expressed by a +1 translational frameshifting at the carboxyl terminus of the polyprotein, which contains two papain-like proteases, a methyltransferase and a helicase domain encoded by OFR1a, and possibly requires proteolytic processing to be cleaved from the polyprotein encoded by ORF1a/1b [20]. Later, it was demonstrated that the putative RdRp of CTV is expressed by a +1 translational frameshift at the carboxyl terminus of the polyprotein [7]. However, the expression of the RdRp of CTV has not been detected in infected plants. The RdRp gene of CTV strain T36 has been expressed as a 60-kDa fusion protein in *E. coli*, and polyclonal antiserum specific for the recombinant RdRp has been raised in rabbit (anti-CTV-RdRp serum) [8]. In this study, expression of the RdRp of CTV was studied *in vivo* and *in vitro* using this anti-CTV-RdRp serum.

For detection of the CTV-RdRp by Western blotting, cell extracts were prepared from *E. coli* strain BL21 with or without the CTV-RdRp gene, and also from 200 to 250 mg of fresh bark tissue from either uninfected citrus tissue or citrus that was infected with CTV strain T36. Proteins were separated by electrophoresis on sodium dodecyl sulfate (SDS)-containing polyacrylamide gels containing

10% acrylamide (SDS-PAGE on 10% polyacrylamide gels) and transferred to nitrocellulose membranes using a semi-dry blotter (Bio-Rad). The membrane was probed with the anti-CTV-RdRp serum produced against the recombinant CTV-RdRp [8], followed by an anti-rabbit secondary antibody conjugated to horseradish peroxidase (HRP). Specific reactions were detected using the Super Signal™ chemiluminescent system for HRP (Pierce). This analysis of the bacterial proteins showed that a protein with a molecular weight of about 60 kDa was detected in *E. coli* transformed with pETh3c-CTVRdRp, but not with pETh3c control (Fig. 1A, lanes 2 and 1, respectively). Analysis of plant extracts from healthy and CTV-infected citrus showed that the antiserum reacted only with CTV-infected citrus and detected a single protein of about 50 kDa; no reaction was observed in the uninfected citrus tissue (Fig. 1, lanes 3 and 4, respectively).

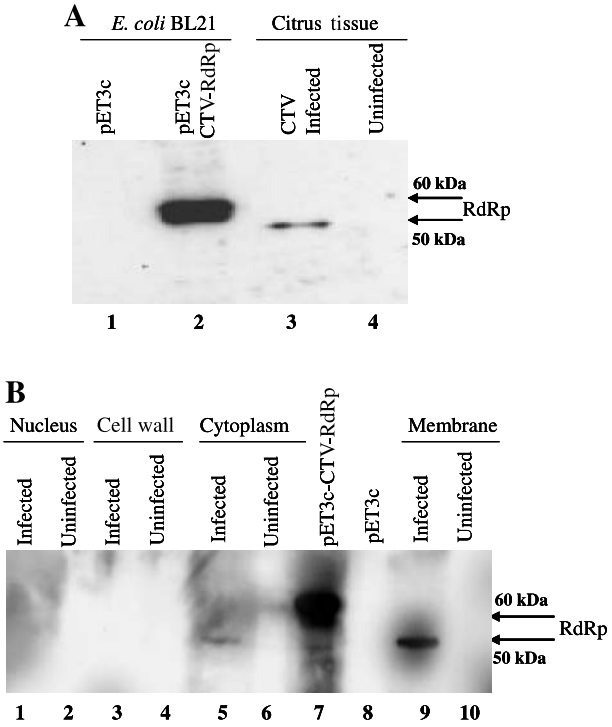

Fig. 1. *In vivo* expression analysis of the RNA-dependent RNA polymerase (RdRp) of Citrus tristeza virus (CTV) by Western blot. (**A**) Detection of the CTV-RdRp expressed in *E. coli* transformed with pETh3c or pETh3c-CTV-RdRp (*1* and *2*) and in CTV-infected and uninfected citrus tissue (*3* and *4*) by Western blot. (**B**) Sub-cellular localization of the CTV RdRp in CTV-infected and uninfected citrus tissue in the nucleus (*1*, *2*), cell wall (*3*, *4*), cytoplasm (*5*, *6*) and membrane fractions (*9*, *10*). *7* and *8* are control samples from *E. coli* transformed with pETh3c-CTV-RdRp and pETh3c, respectively. Samples were separated by SDS-PAGE on 10% polyacrylamide gels, transferred to nitrocellulose membranes, probed with anti-CTV-RdRp serum and horseradish peroxidase-conjugated anti-rabbit antibody, and detected using the Super Signal™ chemiluminescent detection system

To determine the sub-cellular localization of the CTV-RdRp in infected citrus tissue, cell fractions of 5 g of uninfected and CTV-infected citrus bark tissue were prepared by differential centrifugation using a previously reported protocol [12]. About 10% of each fraction from infected and uninfected tissue samples was analyzed by western blot using anti-CTV RdRp serum as described above. As controls, the 60-kDa recombinant RdRp protein was detected in cell lysate from *E. coli* transformed with pETh3c-CTV-RdRp but not with pETh3c (Fig. 1B, lanes 7 and 8). A 50-kDa protein was detected in the membrane and cytoplasmic fractions of the CTV-infected tissue (Fig. 1B, lanes 5 and 9), but no reactions were observed with any fractions of uninfected tissue (Fig. 1B, lanes 2, 4, 6, and 10). The protein was present in the cytoplasmic fraction, but it accumulated at a much higher level in the membrane fraction of the infected cells, suggesting that this protein is associated with membranes, as are the RdRps of other RNA plant viruses [17].

Interestingly, the 50-kDa protein detected in CTV-infected tissue was smaller than the putative 57-kDa CTV-RdRp encoded by ORF1b, and the CTV polyprotein, which is estimated to be 400 kDa. To examine this discrepancy, to study the expression of the RdRp *in vitro*, and to further confirm the specificity of the antiserum, the pETh3c and pETh3c-CTV-RdRp [8] were transcribed and translated using the TNT *in vitro* transcription and translation kit (Promega) with T7 polymerase in rabbit reticulocyte extract and labeled with ^{3}H leucine for 1.5 h at 30 °C according to the manufacturer's instructions. A portion of the translation product was used for analysis of total protein. Analysis of the translation products showed that the expected 60-kDa protein as well as a protein of 50 kDa and a small protein of about 10 kDa were produced from

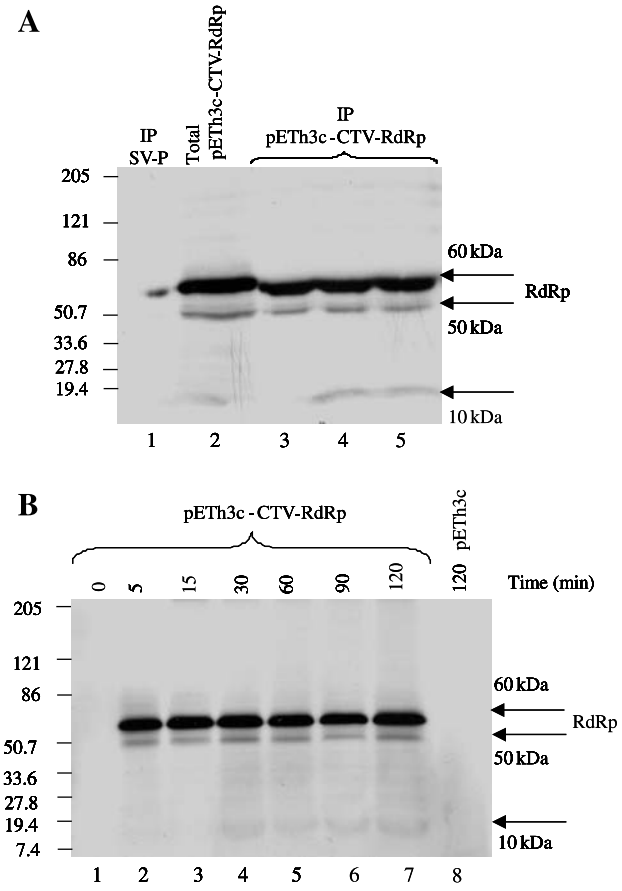

Fig. 2. *In vivo* expression analysis of the RNA-dependent RNA polymerase (*RdRp*) of Citrus tristeza virus (*CTV*). (**A**) Analysis of CTV-RdRp samples expressed from pETh3c-CTV-RdRp by *in vitro* transcription and translation in rabbit reticulocyte lysate and immunoprecipitation with anti-CTV-RdRp serum. *1* is the phosphorprotein of Sendai virus (unrelated protein used as negative control). *2* is a sample analyzed directly following transcription and translation of pETh3c-CTV-RdRp, and lanes *3–5* are samples immunoprecipitated with the CTV-RdRp antiserum. (**B**) Analysis of CTV-RdRp samples expressed from pETh3c-CTV-RdRp or pETh3c by *in vitro* transcription and translation in the rabbit reticulocyte lysate. *2–7* are samples taken after various times of transcription and translation of plasmid pETh3c-CTV-RdRp. *1* is the 0 time control translation product. *8* is a negative control sample taken after 120 min of transcription and translation of pETh3c. Samples were separated by SDS-PAGE on 10% polyacrylamide gels, transferred to nitrocellulose membranes, probed with anti-CTV-RdRp serum and horseradish peroxidase-conjugated anti-rabbit antiserum, and detected by the Super Signal™ chemiluminescent detection system

pETh3c-CTV-RdRp (Fig. 2A, lane 2), and no protein was produced from pETh3c (Fig. 2B, lane 8). To determine if these proteins were, in fact, the CTV-RdRp, the remaining translation products were immunoprecipitated with the anti-CTV-RdRp serum. For this, samples were first resuspended in immunoprecipitation buffer [0.15 M NaCl, 50 mM Tris–HCl (pH 8.0) and 1 μg ml^{-1} aprotinin] and incubated with the anti-CTV-RdRp serum for 2 h at 4 °C, then with Protein A beads (Sigma) at 4 °C for 1 h. The beads were washed 3 times with 1% NP-40 buffer [0.15 M NaCl, 50 mM Tris-HCl (pH 8.0), 1% Nonidet P-40 (NP-40), and 1 μg ml^{-1} aprotinin] and resuspended in 2× lysis buffer [4% SDS, 100 mM Tris-HCl (pH 6.8), 200 mM DTT, 20% Glycerol and 0.1% bromophenol blue]. Total translation products and immunoprecipitated proteins were separated by SDS-PAGE on 10% polyacrylamide gels and detected by autoradiography. As shown in Fig. 2A, lanes 3, 4 and 5, all three proteins, 60, 50 and 10 kDa, produced from pETh3c-CTV-RdRp expressing the 60-kDa fusion protein were immunoprecipitated with anti-CTV-RdRp serum. Furthermore, when a plasmid expressing the unrelated Sendai virus phosphoprotein was used as negative control, no phosphoprotein was immunoprecipitated (Fig. 2A, lane 1). This demonstrates that the 50-kDa and 10-kDa proteins were likely fragments of the 60-kDa CTV-RdRp fusion protein, and it also demonstrates the specificity of the anti-CTV-RdRp serum. This suggested that the expected 60-kDa protein was expressed initially *in vitro* in the rabbit reticulocyte lysate, and that the 50- and 10-kDa proteins probably resulted from further processing of that 60-kD protein.

To test the possible processing of the 60-kDa protein, a time course experiment was performed by translating the pETh3c-CTV-RdRp and labeling for 5, 15, 30, 60, 90 and 120 min in rabbit reticulocyte lysate. Translation was stopped by adding of an equal volume of 2× lysis buffer, and the translation products were separated by SDS-PAGE on 10% polyacrylamide gels and detected by autoradiography. Similar amounts of the 60-kDa unprocessed protein and the 50-kDa processed proteins were detected at all time points (Fig. 1B, lanes 1–6), whereas a small amount of the 10-kDa protein

was detected starting at 30 min and continuing up to 120 min (Fig. 2B, lanes 4–7).

It has been proposed and previously demonstrated for CTV that the RdRp is expressed via a +1 translational frameshift at the carboxyl terminus of its polyprotein [7, 20]. However, the protein itself had not been detected *in vivo*, and the location and fate of the RdRp in infected citrus tissue was unknown. Western blot analyses of extracts of CTV-infected plants demonstrated the presence of the 50-kDa protein and that it was localized primarily in the membrane fraction. This is consistent with the subcellular localization of the RdRp and other replication-associated proteins of other RNA viruses [17], suggesting that this protein is likely to be the RdRp of CTV.

These results also revealed potentially significant information about the expression and fate of the CTV-RdRp in infected citrus. First, the detection of the RdRp *in vivo* is consistent with the proposal [20] and our initial findings that the CTV RdRp is expressed via a +1 translational frameshift [7]. Second, the CTV-RdRp is possibly cleaved from the CTV polyprotein and expressed as an individual protein in CTV-infected tissue. And third, the cleavage of the CTV-RdRp from the polyprotein likely occurred a site downstream of the proposed frameshift site [20], resulting in a smaller-than-expected protein in CTV-infected citrus tissue. This was supported by detection of the 50-kDa protein rather than the expected 57-kDa RdRp or the 400-kDa CTV-polyprotein. This processing was mimicked to some degree in the rabbit reticulocyte lysate system, where the 60-kDa primary product was partially processed into 50-kDa and 10-kDa products, both of which were immunoprecipitated by the antiserum specific for the protein expressed from the CTV RdRp gene.

It was reported for beet yellows virus (BYV), the type virus of the genus *Closterovirus*, that the helicase and methyltransferase domains of the polyprotein were detected as individual proteins in BYV-infected tissue, suggesting that these proteins were cleaved from the BYV polyprotein by viral or host proteases [9]. The polyprotein of BYV was not detected by antibodies specific for the helicase and methyltransferase domains, which are expressed as part of the polyprotein [9]. Therefore, our results for CTV are consistent with previous reports on the expression of closterovirus replication-associated proteins. They also support the emerging concept that the polyprotein of viruses in the genus *Closterovirus* is not active as a single large polyprotein with many functions, but that it is processed and cleaved into individual functional domains to accomplish their specific functions [9]. The site(s) of cleavage in the polyprotein and the nature and the origin of the protease(s) responsible for processing the polyprotein are still unclear. The absence of processing of the CTV-RdRp in *E. coli* suggests that the protein is cleaved by viral or eukaryotic cellular proteases. Detection of the processed RdRp both *in vivo* and *in vitro* in rabbit reticulocytes, where no viral genome is present, suggests an involvement of cellular proteases. A host cysteine-like protease processing the RdRp of Leishamania virus from its polyprotein precursor has been identified [30]. Although this virus has a dsRNA genome and is taxonomically unrelated to CTV, both viruses use the +1 translational frameshifting mechanism for the expression of their RdRp.

Despite significant advances in the molecular characterization of CTV in resent years [1, 2 14–16, 20, 23–25, 28, 31, 33, 34, 36, 37], this appears to be the only study related to the RdRp of CTV or any other closterovirus. It reports detection of the CTV-RdRp in infected citrus tissue and provides findings to aid in understanding the expression of the CTV-RdRp. However, further and more comprehensive studies are needed to completely elucidate CTV-RdRp expression and function during the infection process.

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